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ORIGINAL ARTICLE

Resistance to *Fusarium oxysporum* f. sp. *gladioli* in transgenic *Gladiolus* plants expressing either a bacterial chloroperoxidase or fungal chitinase genes

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Abstract Three antifungal genes, a non-heme chloroperoxidase from *Pseudomonas pyrrocinia*, and an exochitinase and endochitinase from *Fusarium venetanum* under regulation by the CaMV 35S promoter, were used to transform *Gladiolus* for resistance to *Fusarium oxysporum* f. sp. *gladioli*. *Gladiolus* plants were confirmed to be transgenic by Southern hybridization. Semi-quantitative RT-PCR of RNA isolated from leaves and roots demonstrated expression of the *Fusarium* exochitinase and endochitinase genes in transgenic plants compared to controls. All transgenic plants expressing the *Fusarium* exochitinase or endochitinase gene had chitinase activity higher than that of the control plants. Semi-quantitative RT-PCR verified that three of the four plant lines with the chloroperoxidase gene expressed the transgene in leaves and roots while no expression was detected in control plants. Western hybridization confirmed the presence of the chloroperoxidase protein in both leaves and roots of transgenic plants. Cell extracts from one endochitinase plant line inhibited growth of germinated *F. oxysporum* spores more consistently than extracts from the four chloroperoxidase and three endochitinase plant lines. Three chloroperoxidase, two exochitinase, and three endochitinase transgenic plant lines sustained a significantly ($P < 0.05$) lower density of hyphae on roots compared to roots of non-transformed *Gladiolus* plants three to four days following exposure of the roots to *Fusarium*. Shoots from two plant lines, one containing a chloroperoxidase and the other an endochitinase gene, had less necrosis when rated on a scale of 1–3 and appeared visually to be healthier and without obvious *Fusarium* infection than non-transformed, regenerated *Gladiolus* plants 17–21 days following exposure to *F. oxysporum*.

Keywords Floral monocots · Stable transformation · Fungus resistance

Introduction

Gladiolus is a popular horticultural crop throughout the world. A major pathogen of *Gladiolus* is the soilborne fungus *Fusarium oxysporum* f. sp. *gladioli* (*Fog*) that can persist in the soil for long periods in the form of chlamydospores (Wilfret 1992). In the past, *Fusarium* was controlled using methyl bromide, but this broad-spectrum fumigant has been phased out for application to soil due to health and environmental concerns, and effective alternatives other than planting in a new location where *Fusarium* is not well established are not currently available. Genetic resistance offers a sustainable alternative to the nursery and bulb industry for control of *F. oxysporum* f. sp. *gladioli*.

Two fungal chitinases and a bacterial chloroperoxidase gene were selected as potential antifungal agents in the current study. Filamentous fungi are composed of at least 20 % chitin that is located in the inner layer of the cell wall adjacent to the plasma membrane of the hyphae (Hartl et al. 2012). All filamentous fungi have a variety of chitinases, and there are three chitinase subgroups characterized by their amino acid sequences, the structure of their substrate-binding region, and their enzymatic activities (exochitinase

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or endochitinase). Endochitinases cleave the internal β-1,4-glycoside bonds of chitin resulting in the release of a mixture of soluble oligomers, and exochitinases cleave the β-1,4-glycoside bonds of chitin releasing only diacetylchitobioses (Cletus et al. 2013). Exochitinases and endochitinases differ in the way they bind their carbohydrate substrates, but this mechanism is not clearly defined and requires additional research (Hartl et al. 2012). In fungi, chitinases function during autolysis of old hyphae, breakdown of wall components during tip expansion, and in nutrient acquisition of saprophytic fungi.

Chitinases have been isolated from *Trichoderma* sp. and shown to confer resistance to the soilborne fungus *Rhizoctonia* in transgenic tobacco and cotton plants and to *Botrytis cineria* in transgenic lemon plants (Dana de las Mercedes et al. 2006; Distefano et al. 2008; Emani et al. 2003; Gentile et al. 2007). Although an exochitinase isolated from *Trichoderma* was effective against *F. oxysporum* in a well diffusion assay, transgenic cotton plants expressing a chitinase from *Trichoderma* did not show resistance to *F. oxysporum* (Emani et al. 2003; Kumar et al. 2011). The exochitinase and endochitinase genes isolated from *Fusarium venetanum* and used in this study were previously shown to endow several lines of transgenic *Triticum aestivum* plants with moderate tolerance to *Rhizoctonia* and *Pythium* as determined by a decrease in disease severity (Okubara et al. 2014).

Another gene chosen for this study to test for potential activity against *Fusarium* was a nonheme chloroperoxidase isolated from *Pseudomonas pyrrocinia* that has brominating activity but not peroxidase activity (Wolfgang et al. 1986; Wiesner et al. 1986). Fungal chloroperoxidases contain protoporphyrin IX as the prosthetic group (van Pée 1996). Halogenated compounds produced by bacteria are secondary metabolites produced late in the log phase or stationary phase. Transgenic tobacco plants expressing this bacterial nonheme chloroperoxidase were found to have resistance to *Aspergillus flavus*, *Colletotrichum destructivum*, and *Alternaria alternata* (Rajasekaran et al. 2000; Ruhlman et al. 2014). Cell extracts from these transgenic tobacco plants prevented colony formation from germinated spores of *A. flavus*, *Fusarium verticillioides*, and *Verticillium dahlia* (Ruhlman et al. 2014). *Arachis hypogaea* (peanut) plants with this transgene showed resistance to *A. flavus* (Niu et al. 2009). Although this nonheme chloroperoxidase has demonstrated antifungal activity in transgenic plants, studies have shown that the peroxidative activity and the generation of peracetic acid do not appear to be the basis for the antifungal activity (Jacks et al. 2000, 2002) suggesting that the brominating activity is important.

This study reports the development of transgenic *Gladiolus* plants containing either an exochitinase, endochitinase, or chloroperoxidase gene, and the antifungal effect of these transgenes on *F. oxysporum* sp. *gladioli* examined. Most antifungal genes isolated from fungi have been chitinases isolated from the biocontrol fungus *Trichoderma* making it interesting to test a chitinase from a fungus other than *Trichoderma* for resistance *in planta.* This is the first report these three genes have been tested *in planta* for their effectiveness against *F. oxysporum*.

Materials and methods

Plants, callus, and suspension cells

Plants of *Gladiolus* cv. Peter Pears were grown in vitro on Murashige and Skoog's (MS) medium (Murashige and Skoog 1962) containing 3 % sucrose, 1.0 mg/L glycine, 1.0 mg/L thiamine, 0.5 mg/L pyridoxine, 0.5 mg/L nicotinic acid, and 0.2 % Phytagel (Sigma-Aldrich, St. Louis, MO, USA), pH 5.8. Embryogenic callus was initiated by growing the plants in vitro on MS medium supplemented with 2.2 μM 2,4-dichlorophenoxyacetic acid (2,4-D). After 2–3 months, the callus that originated from the plant's basal meristem was removed and cultured in the dark on MS medium containing 2.2 μM 2,4-D to multiply the callus. Callus that was friable was placed in 30 mL of liquid MS medium supplemented with 2.2 μM 2,4-D in a 125 mL flask and shaken at 120 rpm on a gyratory shaker to initiate cell suspensions. Cell suspensions that were 3–4 months old were used for gene gun bombardment.

Plants, callus, and suspension cells were transferred every 3–4 weeks to fresh medium and grown at 25 °C. Plants were grown under a 12 h light photoperiod using cool white fluorescent light bulbs (40–60 μmol/m^2/s). Callus and suspension cells were grown in the dark.

Plasmids

The 845 bp chloroperoxidase gene (Wolframm et al. 1993) was subcloned under the CaMV 35S promoter in pBS-35S CaMV-CPO-rbcS (Fig. 1). Both the 1.4 kb exochitinase and 1.15 kb endochitinase genes were isolated from *F. venetanum* and subcloned under the double CaMV 35S promoter in psan14 (received from Sanford Scientific, Inc., Waterloo, NY, USA). Selection in plants was achieved using p35SAc (received from AgrEvo, Somerville, NJ, USA) that contains the phosphinothricin acetyltransferase gene under control of the CaMV 35S promoter.

Transformation

Plasmid DNAs were isolated from *E. coli* DH5α by alkaline lysis and purified on a cesium chloride gradient (Maniatis et al. 1982). Suspension cells of *Gladiolus* were co-

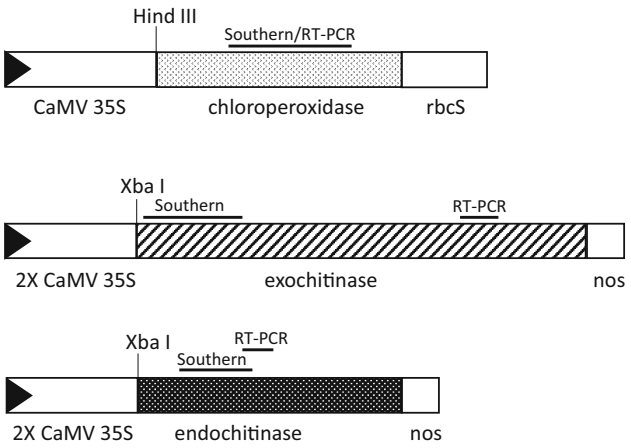

Fig. 1 Diagram of DNA constructs used for transformation of *Gladiolus* 'Peter Pears.' The region of the transgene amplified by PCR used as a probe during either the Southern hybridization or RT-PCR is indicated by a *line above the transgene*

bombarded with equal amounts of plasmid DNA (p35SAc and an antifungal plasmid DNA). Gold particles (0.75 μm) were coated with plasmid DNA according to the method of Sanford et al. (1993). Suspension cells were grown for 4 h in MS medium supplemented with 2.2 μM 2,4-D and 0.125 M mannitol before being plated as a layer on a Whatman no. 4 filter paper disk that was placed on solid MS medium containing 0.125 M mannitol and 2.2 μM 2,4-D. Each plate of cells was bombarded once at 1200 psi using the PDS-1000/Helium gene gun (Bio-Rad, Hercules, CA, USA) that had a 1 cm gap and 1 cm flying membrane distance with a 12 cm target distance. After bombardment, the cells were transferred to MS medium supplemented with 2.2 μM 2,4-D solidified with 0.2 % Phytagel to promote growth of the cells.

Bombarded suspension cells were transferred to MS medium containing 2.2 μM 2,4-D and 0.1 mg/L bialaphos (Meiji Seika Kaisha, Tokyo, Japan) 1 week after bombardment. One month later, cells were transferred to the same medium with an increased bialaphos concentration (1.0 mg/L). Cells were grown at 25 °C in the dark and transferred monthly to fresh medium. Small plantlets that regenerated during this time were transferred to MS medium supplemented with 0.1 mg/L bialaphos and grown in the light under a 12 h light photoperiod. Larger plantlets were grown on MS medium supplemented with 1.0 mg/L phosphinothricin (AgrEvo). Non-transformed plants regenerated from the suspension cells served as the control.

Southern hybridization

Genomic DNA was isolated from leaves of *Gladiolus* plants grown in vitro using the method of Dellaporta et al. (1983) for Southern hybridization. DNA from leaves transformed with the chloroperoxidase and chitinase genes were digested with *Hin*dIII and Xba I, respectively, and restriction fragments separated by electrophoresis using a 1 % agarose gel in TBE buffer (9 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). Digested DNA was transferred to a Nytran nylon membrane (Schleicher-Schuell, Keene, NH, USA) by capillary movement (Maniatis et al. 1982).

PCR amplification of plasmid DNA was used to prepare probes for Southern hybridization (Fig. 1). Primers used for amplification of a 545 bp product from the chloroperoxidase gene were sense 5′-GCTCTTCTTCGTTCAGAAGGGC-3′ and antisense 5′-GCACGAGCGTCGGGACGGTGAT-3′. The sense primer 5′-AGCTCTGGCACTTCCTGGTA-3′ and antisense primer 5′-ACATCAGAGCTGTTCATGCG-3′ amplified a 461 bp region of the exochitinase gene. Primers used for amplification of a 427 bp endochitinase gene product were sense 5′-GAACCTCGGTTTTGACGGTA-3′ and antisense 5′-GTCGGTGTTCTCAAAGGCTC-3′. Amplification was achieved using a PTC-200 Microcycler (MJ Research, Waltham, MA, USA) programmed at 94 °C for 2 min, 31 cycles (94 °C for 1 min, 62 °C for 1 min, 72 °C for 1 min), and 72 °C for 10 min. The PCR reaction mixture was electrophoresed on an agarose gel, and the desired PCR product was gel-purifed using a QIAquick gel extraction kit (Qiagen, Inc., Valencia, CA, USA).

Probes for Southern hybridization were labeled with α-[^{32}P]dCTP using the DECAprime II kit (Ambion Inc., Austin, TX, USA). DNA blots were prehybridized in Church's buffer [7 % sodium dodecyl sulfate (SDS), 1 % bovine serum albumin, 1 mM EDTA, and 250 mM $NaPO_4$, pH 7.2] (Church and Gilbert 1984) for 1 h at 42 °C. The radioactive probe was added to the hybridization solution [6× citric acid trisodium salt (SSC), 10 mM EDTA, 5× Denhardt's solution, 0.5 % SDS, 100 μg/mL denatured salmon sperm DNA] (Maniatis et al. 1982) for 16 h incubation at 60 °C with the DNA blot. Blots were washed first in 2X SSC, 0.2 % SDS, then 1X SSC, 0.2 % SDS, and lastly 0.1X SSC, 0.2 % SDS. Each wash was done for 15 min at 55 °C, and then the blot was exposed to BioMax X-ray film (Carestream Kodak, Sigma-Aldrich) with an intensifying screen at −70 °C for 1–5 days.

Semi-quantitative RT-PCR and quantum PCR

RNA was isolated from roots and leaves of in vitro-grown plants using Qiagen's RNeasy kit according to the instruction manual. Reverse transcription of RNA was done using an Invitrogen ThermoScript RT-PCR kit (Invitrogen Life Technologies, www.lifetechnologies.com) to obtain cDNA (20 μL reaction volume containing 1 μL RNaseH). Amplification of cDNA was done using a 25 μL

reaction mixture of 1 μL cDNA, 100 nM each primer, buffer, and 1.5 mM $MgCl_2$. Primers used for amplification of a 545 bp product from the chloroperoxidase gene were sense 5′-GCTCTTCTTCGTTCAGAAGGGC-3′ and antisense 5′-GCACGAGCGTCGGGACGGTGAT-3′. Exochitinase was amplified using the sense primer 5′TGTCGC GTACAATGAGAAGC-3′ and antisense primer 5′-GTACTTGGAAATGCGAGGGA-3′ resulting in a 136 bp product. Endochitinase was amplified using the sense 5′-TGCCAACGAGGCTAGAAACT-3′ and antisense 5′-GGTATTGATCCATTCCACGG-3′ primers to obtain a 167 bp product. The PCR conditions were 94 °C for 2 min, 31 cycles (94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min), and 72 °C for 10 min.

As an internal standard for gene expression, the *Gladiolus grandiflorum* polyubiquitin gene *GUBQ2* (GenBank accession number EU563360) was amplified using the sense primer 5′-ATGCAAATTTTCGTCAAGACCC-3′ and antisense primer 5′-ACCACCACGGAGGCG-3′, and conditions for amplification were as stated previously for semi-quantitative RT-PCR, except that the annealing temperature was 55 °C.

The Quantum RNA™Universal 18S Internal Standards kit (Ambion, www.ambion.com) was used to determine relative amounts of chloroperoxidase RNA in leaves of transgenic plants. The kit's 18S rRNA primers and competimers were added to the PCR reaction in a 4:6 ratio. Primers used for amplification of the chloroperoxidase gene are the same as stated previously for semi-quantitative RT-PCR. RNA was isolated using the RNAqueous kit (Ambion) and DNA-free reagent (Ambion) according to the manufacturer's instructions. PCR amplification was performed at 94 °C for 2 min, 35 cycles (94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min) and 72 °C for 6 min. PCR products were separated by electrophoresis through a 1.5 % agarose gel and visualized following staining with ethidium bromide.

Western blots for chloroperoxidase protein

All solutions, unless specified otherwise, were purchased from Invitrogen Life Technologies. Tissues (200 mg fresh weight) of *Gladiolus* leaves or roots were ground in liquid nitrogen followed by the addition of 0.5 mL extraction buffer (62.5 mM Tris HCl, pH 6.8, 10 % glycerol, 1X Sample Reducing Agent). The extract was centrifuged at 10,000×*g* for 5 min at 4 °C, and the supernatant collected then diluted in an equal volume of NuPage 2× Reducing Sample Solution Buffer. Samples were loaded on a 12 % NuPAGE Novex Bis–Tris 1.0 mm precast acrylamide gel and then electrophoresed using an Invitrogen Novex X-Cell Sure Lock gel electrophoresis system for 90 min at 150 volts in MOPS SDS Running Buffer according to the manufacturer's instructions. Proteins were visualized following staining in Simply Blue Safe Stain for 16 h and destained in water for 60 min.

The chloroperoxidase antibody solution was cross-absorbed with healthy tissue extract to reduce non-specific binding of the chloroperoxidase polyclonal antibody (gift from K.-H. van Pée). Non-transformed leaf tissue of *Gladiolus* (1.5 g fresh weight) was ground in liquid nitrogen, incubated with 10 mL water at 25 °C for 5 min, then 10 mL of 1X maleic acid tween (MAT) buffer added for 5 min, followed by centrifugation at 12,000×*g* for 10 min. The supernatant was filtered through cheesecloth, and 17 mL of supernatant transferred to a new tube. Tris buffered saline (TBS) (14 mL) was added to the extract. Just prior to use, the chloroperoxidase polyclonal antibody was diluted 1:200 in this healthy tissue extract and incubated for 1 h at 25 °C before using directly on the membrane as noted below.

Proteins were electroblotted from the gel to an Invitrogen PVDF membrane using the Novex X-Cell SureLock blotting system and NuPAGE Transfer Buffer containing 10 % methanol according to the manufacturer's instructions for Western blotting. The membrane was then placed in BBR-1X MA blocking solution (1:5 dilution of Roche DIG Wash and Block Buffer Set Blocking solution in 1× maleic acid) (Roche Life Science, lifescience.roche.com) for 60 min followed by incubation with the chloroperoxidase antibody (1:200 dilution) that had previously been cross-absorbed with healthy tissue. After incubation for 16 h at 4 °C with the cross-absorbed primary chloroperoxidase antibody, the membrane was washed 3X, 10 min each wash, in 1× MAT (maleic acid plus tween; Roche DIG Wash and Block Buffer Set). The membrane was then incubated for 3 h at 25 °C in anti-rabbit alkaline phosphatase conjugate (diluted 1:3333 in BBR-1X MA) while shaking at 50 rpm. This was followed by four washes, 15 min each wash, in 1× MAT buffer. Prior to addition of substrate solution, the membrane was washed 10 min in Detection Substrate Buffer (100 mM Tris, 100 mM NaCl, 10 mM $MgCl_2$, pH 9.5). The membrane was then incubated in the 5-bromo, 4-chloro, 3-indolylphosphate (BCIP) and nitroblue tetrazolium (NBT) substrate solution (Kierkegaard & Perry Laboratories, Gaithersburg, MD, USA). The reaction was terminated by decanting the BCIP-NBT substrate solution, followed by two brief rinses in water.

Chitinase activity assay

Cell extracts were prepared by placing 0.1 g leaf tissue from *Gladiolus* plants with 350 μL of extraction buffer (0.1 M citric acid monohydrate, pH 3) in a Lysing Matrix tube containing one ceramic bead and homogenizing for 15 s using the FastPrep system (QBiogene, Carlsbad, CA,

USA). Chitinase activity was determined using a Fluorometric Chitinase Assay kit (Catalog number CS1030, Sigma-Aldrich) according to the manufacturer's directions. The substrates were 4-methylumbelliferyl β-D-glucosamide and 4-methylumbelliferyl β-D-N,N′,N″-triacetylchiotriose for measuring exochitinase and endochitinase activity, respectively. A standard curve was generated using the chitinase stock from *Trichoderma* provided with the kit. Each sample within each biological replicate had two technical replicates. There were three biological replicates for each sample. A one way ANOVA followed by a Holm-Sidak test (Systat, www.systat.com) was used to determine if the chitinase activity from transgenic plant lines was significantly different than that of non-transformed, regenerated plants at the $P < 0.05$ level of significance.

Inhibition of *Fusarium oxsporum* f. sp. *gladioli* colony forming units by plant extracts

Gladiolus leaves (1 g fresh weight) were ground in liquid nitrogen using an autoclaved mortar and pestle followed by the addition of 1 mL of 10 mM phosphate buffer, pH 5.5. Plant extracts were centrifuged at 10,000×*g* for 5 min at 4 °C, and the supernatant (450 µL) was placed in a new microcentrifuge tube with 25 µL of a *Fog* spore suspension (1×10^5/mL). *Fog* spores had been germinated by incubating in Potato Dextrose broth for 8 h at 30 °C. A 100 µL aliquot of the *Fog* spore and plant extract mixture was spread out in a Petri plate containing Potato Dextrose agar medium, and plates were incubated at 25 °C for 2 days at which time the number of *Fog* colony forming units that had formed mycelial colonies were counted. Each replicate consisted of three plates, and there were four biological replicates for each sample. A one way ANOVA followed by either a Holm-Sidak or Tukey test depending on the recommendation by Systat were used to determine if the plant extract from transgenic plant lines significantly affected colony forming units of *Fog* as compared to non-transformed, regenerated plant extracts at the $P < 0.05$ level of significance.

Challenge of transgenic plants in vitro

Young *Gladiolus* plants were grown in Petri plates (150 × 15 mm) in water agar (0.7 % wt/vol Phytoblend) (Caisson Labs, www.caissonlabs.com). Plates were incubated under a 12 h light photoperiod at 25 °C for 3 days before inoculating with a 5 mm plug taken from a Petri plate containing actively growing *Fog* on Potato Dextrose Agar. A plug of *Fog* was placed at a 1 cm distance from a root of each *Gladiolus* plant, and the roots were observed 3–4 days following inoculation with *Fog*. Plants were stained with lactophenol cotton blue (Larone 1995) to visualize *Fog* hyphae under a dissecting microscope. The degree of infection was given a value: 1-few, relatively short hyphae covering approximately 10–25 % of the root area, 2-moderate amount of hyphae covering approximately 30–50 % of the root area, 3-considerable amount of hyphae appearing to cover the majority (approximately 70 % and more) of the root area, and hyphae are longer in length than seen in roots with a number 2 rating (Fig. 8). A minimum of six roots were evaluated for each replicate. There were three biological replicates to collect data from at least 18 roots/plant line. The value for each transgenic plant line was compared to that of non-transformed, regenerated plants of *Gladiolus* 'Peter Pears' using a student's t test. Lines with different letters are significant at $P < 0.05$.

Shoots of the *Gladiolus* plants were observed daily for 21 days after the plug of *Fog* had been placed 1 cm from the roots. Each day the plant was given a value indicating the degree of *Fog* infection: 3-plant shoot appears healthy and has green leaves, 2-*Fog* apparently present at the shoot/root junction region and necrosis at that region, 1-shoot either covered with *Fog* or *Fog* beginning to grow out of the leaves. Four shoots were observed for each replicate. There were three biological replicates to collect data for 12 shoots/plant line. Data used for analysis was taken 12–21 days after infection depending on when the majority (>60 %) of the non-transformed, regenerated *Gladiolus* plants were first judged to have a value of 1. The value assigned each transgenic plant line was compared to that of non-transformed, regenerated plants of *Gladiolus* 'Peter Pears' using a one way ANOVA followed by a Mann–Whitney Rank Sum Test. Significance was determined at the $P < 0.05$ level.

Results

Integration of transgenes

Stable integration of the chloroperoxidase, endochitinase, or exochitinase transgenes in the *Gladiolus* genome was confirmed following Southern hybridization of DNA blots using a probe specific for a region of either the chloroperoxidase, endochitinase, or exochitinase gene (Figs. 1, 2). Genomic DNA from plants with the chloroperoxidase gene was digested with *Hin*dIII, and DNA from plants with an exochitinase or endochitinase gene with Xba I (Fig. 1) resulting in a single cut within the transgene region. The sizes of the hybridizing bands were unique for each transformant indicating that each plant line was an independent transformation event. Transgenic plant lines 79 and 138 contained a single copy of the

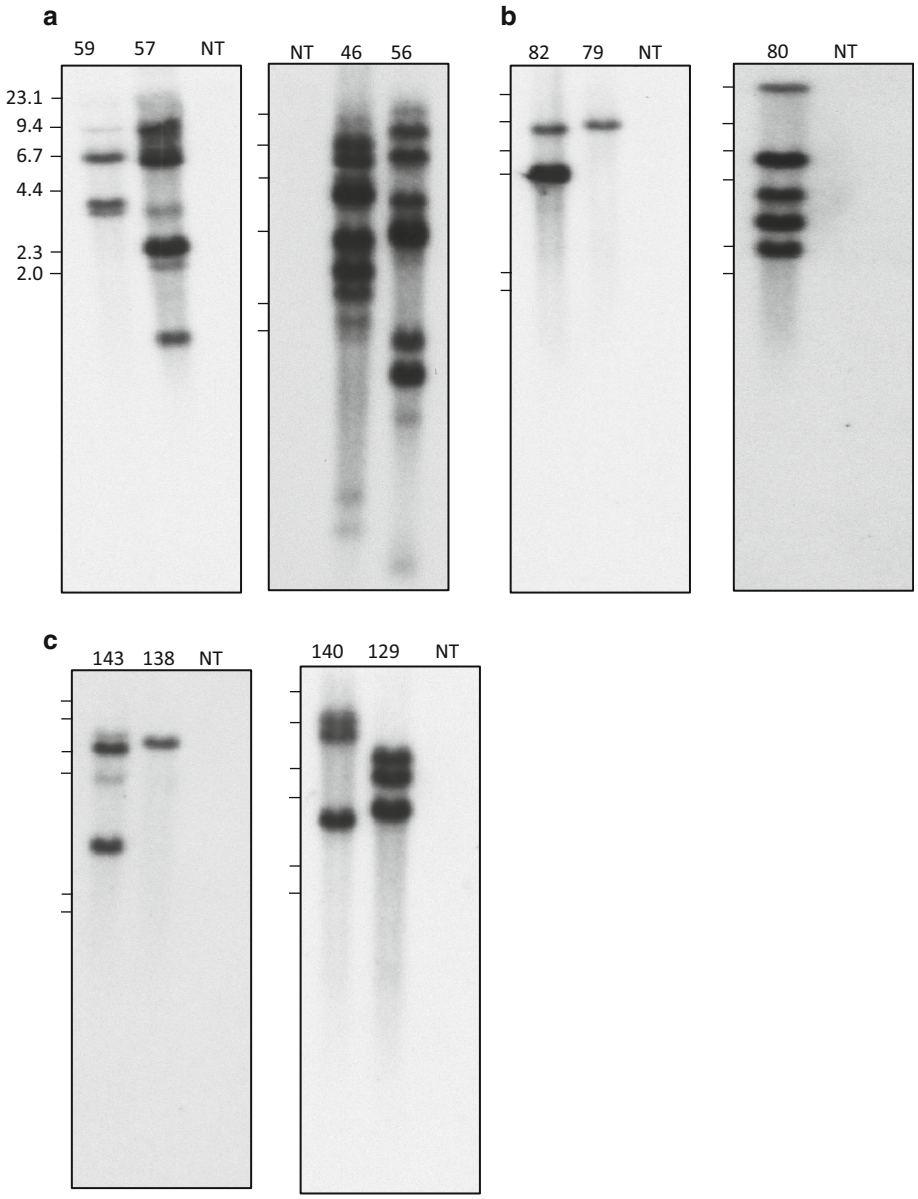

Fig. 2 Stable integration of the **a** chloroperoxidase, **b** exochitinase, and **c** endochitinase transgenes in the *Gladiolus* genome was confirmed by Southern hybridization of DNA blots. Genomic DNA from the **a** chloroperoxidase plants was cut with *Hin*dIII, and DNA from **b** exochitinase and **c** endochitinase plants with Xba I. DNA blots had 20 μg DNA/lane and were hybridized with probes labeled with α-[^{32}P]dCTP that were specific to a region within each transgene located within the coding region. Molecular weight markers indicated on *left* and shown in kb are the same for all DNA blots shown

exochitinase or endochitinase gene. Six plant lines contained 2–4 copies, and the other three lines had at least 6–7 copies of the transgene.

Transgene expression, western blot, and chitinase activity assays

The four transgenic lines of *Gladiolus* containing the chloroperoxidase gene varied in chloroperoxidase gene expression (Fig. 3a). The first plants that regenerated from selection medium were shown to express chloroperoxidase using Quantum PCR analysis. Because Quantum PCR was found not as reproducible as semi-quantitative RT-PCR, RT-PCR was later used for the transgenic plants that had been in culture several years. Line 59 showed the highest level of expression of the chloroperoxidase gene in both leaves and roots as determined by semi-quantitative RT-PCR. In comparison line 56 showed no expression in leaves and negligible expression in roots. Line 46 showed much higher expression of chloroperoxidase in leaves than roots. Plant line 57 expressed chloroperoxidase in roots, and expression was variable in leaves. Production of the chloroperoxidase protein confirmed the transgene expression data (Fig. 4a). Leaves of plant lines 46, 57, and 59 showed the presence of a 31 kD protein following western blotting with a polyclonal antibody to chloroperoxidase (Fig. 4a). Because plant line 59 had shown the highest level of chloroperoxidase gene expression by RT-PCR, it was selected to confirm expression of the chloroperoxidase protein in roots (Fig. 4b).

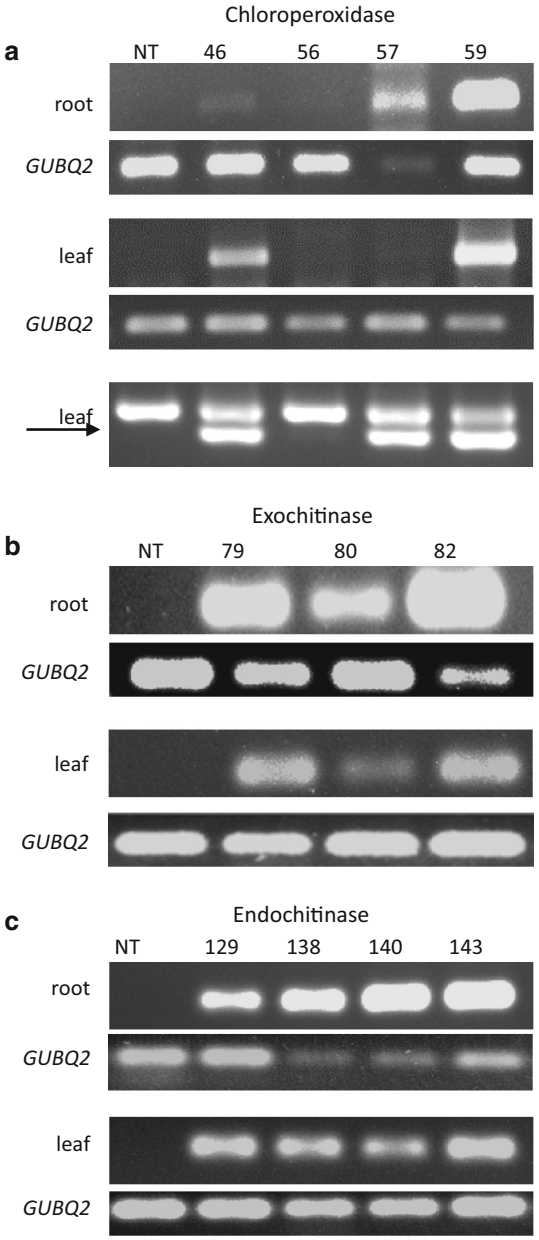

Fig. 3 Semi-quantitative RT-PCR was used to confirm the RNA expression level of either the (**a** top root and middle leaf panels) chloroperoxidase, **b** exochitinase, or **c** endochitinase genes in both roots and leaves of *Gladiolus* plants. Relative levels of RNA expression was compared using Quantum RNA PCR (**a** lower panel with chloroperoxidase RNA indicated by the *arrow*) in leaves from plant lines containing the chloroperoxidase gene. Amplification of the *Gladiolus grandiflorus* polyubiquitin *GUBQ2* gene was used as the RNA internal control

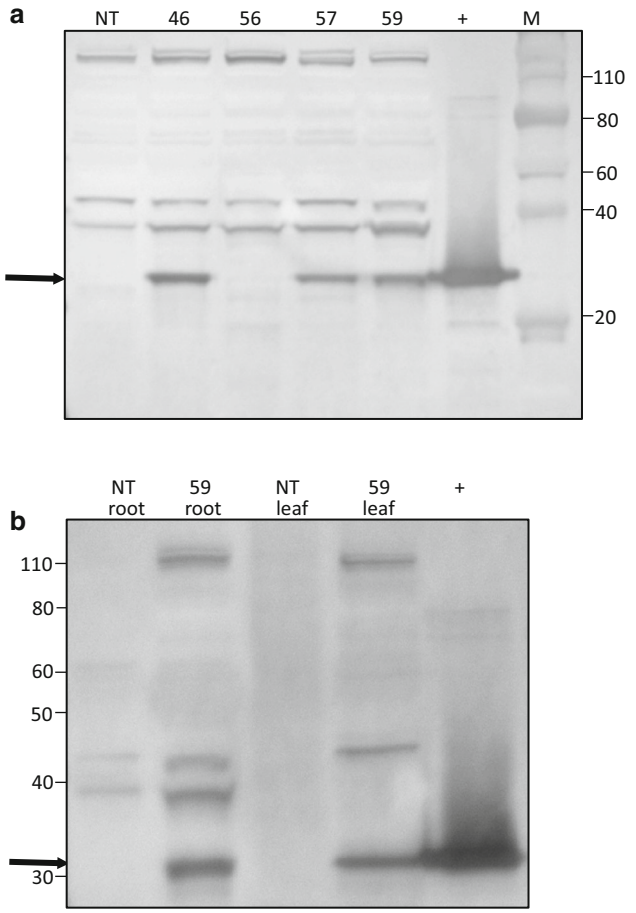

Fig. 4 Western blot showing the presence of the 31 kD chloroperoxidase protein (*arrow*) in cell extracts from **a** leaves of four transgenic plant lines and **b** roots and leaves of plant line 59. Purified chloroperoxidase protein (10 ng) was the positive control (+). Sizes of the protein marker ladder (M) are indicated in kD

Leaves of all three plant lines (79, 80, and 82) transformed with the exochitinase gene showed expression of the transgene as determined by semi-quantitative RT-PCR (Fig. 3b). Line 82 showed the highest level of exochitinase expression in leaves followed by line 79. Exochitinase expression was higher in roots as compared to leaves. All four plant lines (129, 138, 140, and 143) containing the endochitinase transgene demonstrated expression in both leaves and roots with higher expression in roots than leaves. In leaves the highest level of endochitinase expression was in plant line 143 and the lowest in line 129 (Fig. 3c).

All plant lines containing either chitinase transgene showed chitinase activity (Fig. 5). The enzyme activity from line 140 was significantly higher than endogenous chitinase activity of non-transformed, regenerated plants.

In vitro spore inhibition assays

Growth of germinated *Fog* spores was inhibited by cell extracts from leaves of the four chloroperoxidase plant lines in only two of the four biological replicates (Fig. 6). Cell extracts from the three *Gladiolus* lines containing the

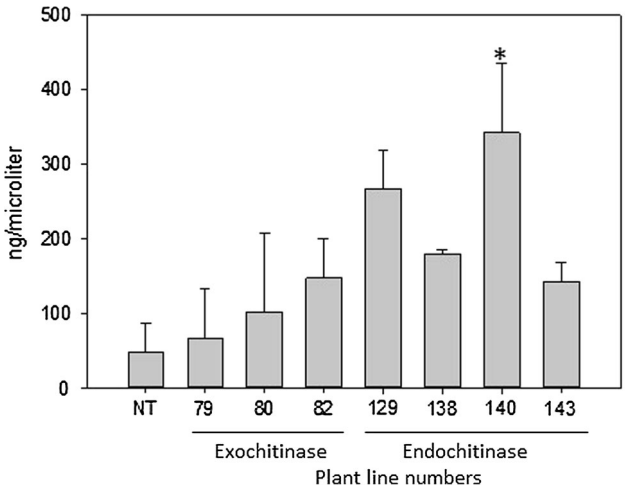

Fig. 5 Chitinase activity in leaves of *Gladiolus* plants containing either the exochitinase or endochitinase gene. *Bars* indicate the mean of three biological replicates with standard *error bars* shown. One plant line with chitinase activity that was significantly different than the non-transformed (NT), regenerated plants at $P < 0.05$ is indicated by a *star above the bar*

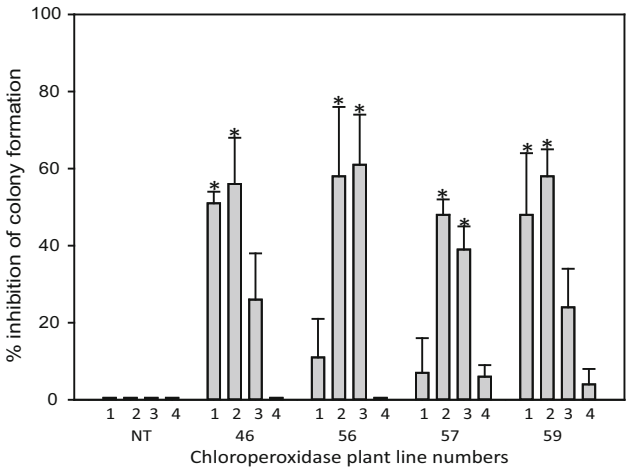

Fig. 6 Inhibition of *Fog* colony forming units by cell extracts from *Gladiolus* plants transformed with the chloroperoxidase gene. Three plates were prepared for each replicate, and four biological replicates done as indicated on the X axis. Plant lines that were significantly different than the non-transformed, regenerated plants at $P < 0.05$ are indicated by a *star above the bar*

exochitinase gene were unable to inhibit the growth of germinated *Fog* spores in at least two of the four replicates (Fig. 7a). In the first experiment, cell extracts from leaves of all four *Gladiolus* lines containing the endochitinase gene showed significant growth inhibition of germinated *Fog* spores, however, only cell extract from line 143 inhibited growth of germinated *Fog* spores in three of the four biological replicates (Fig. 7b). Cell extracts from line 138 inhibited the growth of germinated *Fog* spores in two of the four biological replicates.

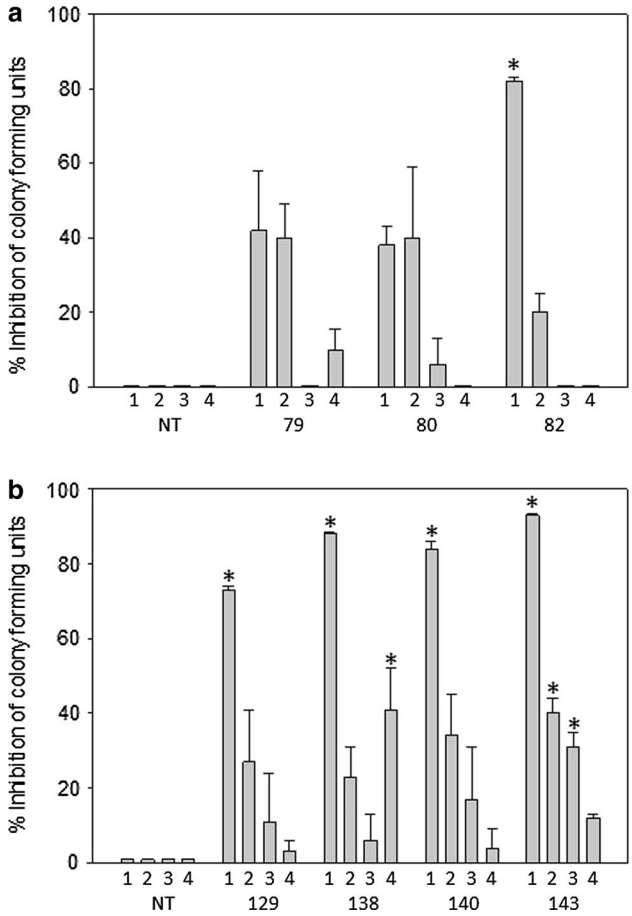

Fig. 7 Inhibition of *Fog* colony forming units by cell extracts from *Gladiolus* plants transformed with either the **a** exochitinase or **b** endochitinase gene. Three plates were prepared for each replicate, and four biological replicates done as indicated on the X axis. Plant lines that were significantly different than the non-transformed, regenerated plants at $P < 0.05$ are indicated by a *star above the bar*

Infection of shoots and roots by *Fusarium oxysporum* f. sp. *gladioli*

Fog infection of the roots of *Gladiolus* plants growing in vitro was evident 3–4 days following inoculation of the water agar that the plant was growing in. At this time, the relative amount of *Fog* infection was judged. Three plant lines (46, 57, and 59) that expressed the chloroperoxidase gene were observed to have less *Fog* present on the roots than the non-transformed, regenerated 'Peter Pears' roots (Figs. 8a, b, 9). Two *Gladiolus* lines (79 and 80) with the exochitinase gene, and three lines (129, 140, 143) with the endochitinase gene were observed to have less *Fog* on their roots than roots of non-transformed, regenerated 'Peter Pears' plants (Figs. 8c, d, 9).

Shoots of the *Gladiolus* plants growing in vitro were also examined for their susceptibility to *Fog* infection. Line 46 with the chloroperoxidase gene and line 129 with the

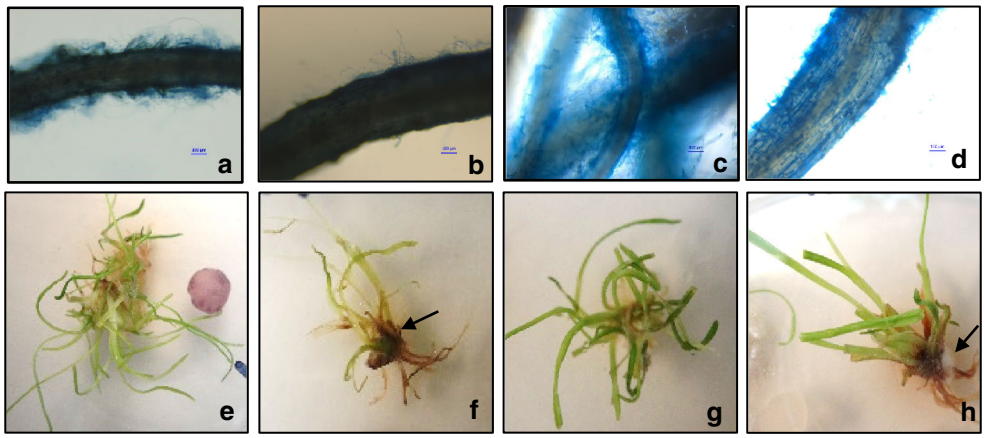

Fig. 8 *Gladiolus* plants 'Peter Pears' grown in vitro and challenged with *Fog*. Roots were challenged with *Fog* and 3–4 days later stained with lactophenol *cotton blue* to enable visualization of *Fog*. The corresponding non-transformed, regenerated 'Peter Pears' plants were challenged in the same replicate as the *transgenic line shown*. Roots of *Gladiolus* **a** non-transformed, regenerated 'Peter Pears', **b** corresponding plant root that contains the chloroperoxidase gene, **c** non-transformed, regenerated 'Peter Pears', and **d** corresponding root that contains the endochitinase gene. Plants of *Gladiolus* **e** line 46 with the chloroperoxidase gene 21 days after inoculation with *Fog*, **f** corresponding non-transformed, regenerated 'Peter Pears' plant 12 days after inoculation showing necrosis at the basal region of the plant (*arrow*), **g** plant line 129 containing the endochitinase gene 12 days after inoculation, **h** corresponding non-transformed, regenerated 'Peter Pears' plant showing necrosis of the basal region with mycelia growing from that region (*arrow*). *Magnification bars* equal 100 μm (**a**–**d**)

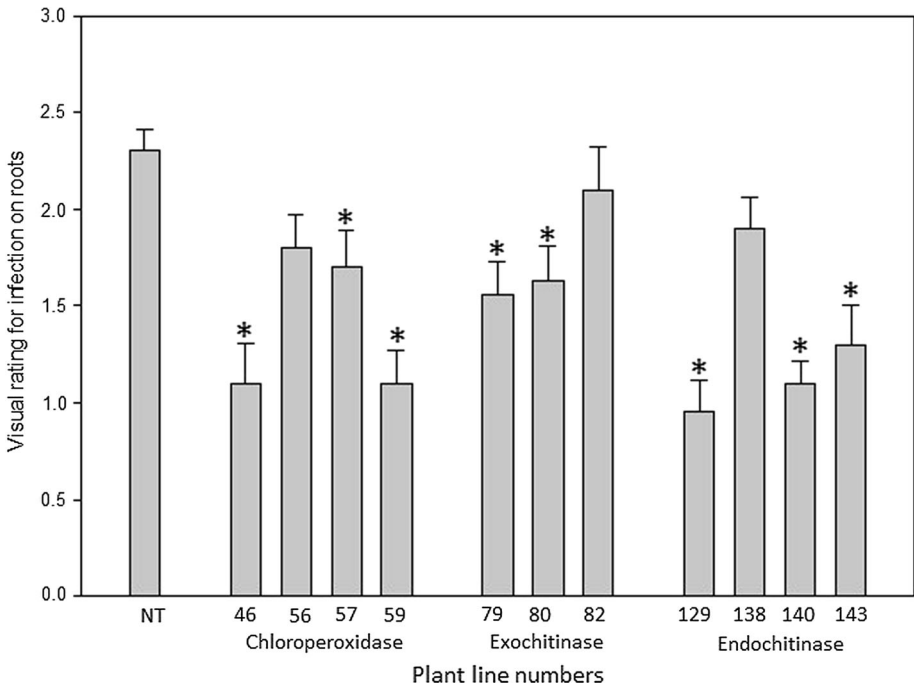

Fig. 9 Roots of *Gladiolus* plants transformed with either the chloroperoxidase, exochitinase, or endochitinase genes were observed 3–4 days following inoculation of the water agar plate with a plug of *Fog* placed 1 cm from the roots. The extent of *Fog* infection was judged visually following lactophenol *cotton blue* staining (1-few hyphae on root, 2-moderate amount of hyphae, and 3-much hyphae present). At least six roots were evaluated for each plant line in one replicate, and three biological replicates were done. Plant lines with a *star above the bar* showed significantly less infection than the non-transformed, regenerated 'Peter Pears' roots at $P < 0.05$

endochitinase gene were observed to have less *Fog* than the non-transformed, regenerated 'Peter Pears' shoots (Figs. 8e–h, 10). Non-transformed, regenerated 'Peter Pears' shoots began to show obvious infection by *Fog* as indicated by the browning at the root/shoot junction region approximately 12 days after infection (Fig. 8f, h). Sometimes a mycelial mass could be seen growing at this region (Fig. 8h). Line 46 plants transformed with the chloroperoxidase gene were observed more often (6/11 plants) to show the highest level of resistance than the other chloroperoxidase plant lines, and sometimes line 46 did not appear to be infected as long as 21 days after inoculation (Fig. 8e). Shoots of the *Gladiolus* plant lines with the exochitinase gene did not appear to be more resistant to *Fog* than the non-transformed, regenerated 'Peter Pears' plants (Fig. 10). Shoots of the endochitinase line 129 were

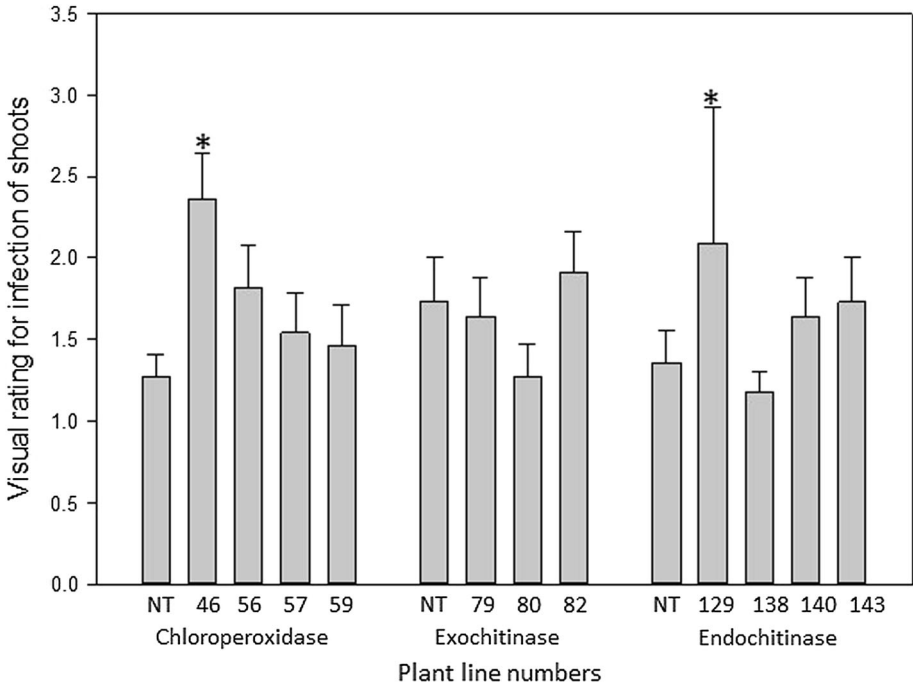

Fig. 10 Shoots of *Gladiolus* plants transformed with either the chloroperoxidase, exochitinase, or endochitinase genes were visually observed up to 21 days following inoculation of the water agar plate with a plug of *Fog* placed 1 cm from roots of the plants. Health of the shoot was judged (1-mycelium is growing from the shoot, 2-shoot is necrotic at the shoot/root region, 3-shoot is green and appears healthy). Shoots from four plants were observed for each replicate, and three biological replicates were done. Plant lines with a *star above the bar* appeared significantly healthier than non-transformed, regenerated shoots at $P < 0.05$

observed to be more resistant to *Fog* infection than the non-transformed, regenerated 'Peter Pears' shoots, and this resistance continued up to 17 days after inoculation in several biological replicates.

Conclusion

Cell extract from one of the *Gladiolus* plant lines containing the endochitinase gene was effective in inhibiting colony formation from germinated spores in vitro whereas cell extracts from both the exochitinase and chloroperoxidase-containing *Gladiolus* plants were ineffective in consistently inhibiting colony formation from germinated *Fog* spores. The shoots and roots of one plant line containing the endochitinase gene and one plant line with the chloroperoxidase gene appeared to be more resistant to *Fog* than the non-transformed, regenerated plants.

Discussion

Cell extract from one transgenic *Gladiolus* plant line (line 143) expressing the endochitinase significantly inhibited colony formation from germinated spores of *Fog* (Fig. 7b). The amount of inhibition (31–93 %) from cell extract of *Gladiolus* plant line 143 transformed with the endochitinase gene was, however, not as effective as cell extracts from *Nicotiana tabacum* transformed with the chloroperoxidase gene (Rajasekaran et al. 2000). Cell extracts from three of the six *N. tabacum* lines showed a 90–100 % inhibition in the number of fungal colony units.

Levels of transgene expression did not always correlate with resistance to *Fog*. It has been found that transgene expression will often change in response to fungal infection so it may have been more appropriate to determine transgene expression when the plants were being challenged with *Fog* (Somssich et al. 1989; Kumar et al. 2009). Another possibility is that enzyme activity, rather than RNA level, is a more accurate indicator of the efficacy of a protein in determining resistance to *Fog*. The exochitinase plant lines were not as resistant to *Fog* as the endochitinase plant lines, and this correlated to chitinase enzyme activity which was higher in all endochitinase plant lines as compared to exochitinase activity (Fig. 5).

It was the goal of this study to transform plants with chitinase genes from a fungus other than *Trichoderma.* Chitinases from *Trichoderma* have been found to be very effective against both a wide range of pathogens and abiotic stress (Lorito et al. 1998; Dana de las Mercedes et al. 2006). *Chi42* expressed in transgenic tobacco and cotton conferred high levels of resistance to *A. alternata and Rhizoctonia solani* (Emani et al. 2003). Chitinases are thought to inhibit fungal growth by digesting their cell wall and also by stimulating fungal elicitors that induce defense reactions within the host (Dana de las Mercedes et al. 2006). Kumar et al. (2009) found that transgenic cotton plants expressing an endochitinase gene from *Trichoderma virens* exhibited a faster and greater induction of reactive oxygen species, expressed several defense-related genes,

and activated certain PR enzymes and the terpenoid pathway following challenge with *Rhizoctonia solani*. Some defense genes were at higher than basal levels in the absence of challenge with *Rhizoctonia* indicating that the expression of the endochitinase appeared to position the plant in a defense mode.

Other strategies for controlling *Fusarium* sp. have involved the use of defensin genes (Ntui et al. 2010; Li et al. 2011; Ghag et al. 2012; Gaspar et al. 2014), synthetic antimicrobial peptides (Chakrabarti et al. 2003; Kamo et al. 2015), lactoferrin (Han et al. 2012), thaumatin (Popowich et al. 2007); apoptosis-related genes (Paul et al. 2011; Ghag et al. 2014), plant-derived chitinases (Li et al. 2001; Shin et al. 2008; Girhepuje and Shinde 2011), a nucleotide binding protein (Wu et al. 2015), an *NPR1* gene (Makandar et al. 2006), a ribosomal protein L3 from yeast (Di et al. 2010), and a pectin methyl esterase inhibitor (Volpi et al. 2011). The only demonstration of *F. oxysporum* resistance in the field was achieved using the *NaD1* defensin isolated from *Nicotiana alata* to transform cotton plants (Gaspar et al. 2014).

The three transgenes in this study resulted in several *Gladiolus* lines that were able to inhibit the growth of *Fog* for a period of time. Jongedijk et al. (1995) showed that transgenic tobacco expressing either a tobacco class I chitinase or a class I β-1,3-glucanase alone did not show resistance to *F. oxysporum* f. sp. l*ycopersici*, however, tobacco plants expressing both transgenes showed a 36–58 % reduction in disease severity when challenged with *Fusarium*. A *Fusarium*-specific antibody coding region was combined with an antifungal peptide gene from *Aspergillus* to make a chimeric antifungal gene, and this used to transform wheat (Li et al. 2008). Results of this study showed a greater reduction in *Fusarium* infection in transgenic plants expressing the antifungal fusion protein than observed in plants expressing the antibody gene alone. The transgenic wheat plants were found to have a lower percentage of their spikelets infected with *Fusarium* head blight. The number of grains produced was higher and the spread of *Fusarium* was restricted in the inoculated spikelets of the transgenic wheat plants as compared to non-transformed plants. This again confirms the importance of combining two genes to effectively confer resistance to *Fusarium*. It is possible that the resistance to *Fog* by *Gladiolus* could have been increased if both the endochitinase and chloroperoxidase genes had been used to transform *Gladiolus.*

Another factor that may have affected the resistance of each transgenic plant line to *Fog* was the evident somaclonal variation amongst the plant lines. The regenerated plants of *Gladiolus* 'Peter Pears' are not as robust as wild type plants, and there were phenotypic differences observed between the roots of each transgenic plant line. Shoots of the two plant lines, 46 and 129 expressing the chloroperoxidase and endochitinase genes, respectively, that were judged to have shoots and roots resistant to *Fog* were also the most robust of the transgenic plant lines so this phenotype may have contributed to their resistance to *Fog*.

Plant pathogens are a significant concern when growing many crops, but only 10 % of the transgenic plants registered for field trials in North America during the last 15 years were engineered for disease resistance (Collinge et al. 2010). This low percentage of transgenic plants engineered for disease resistance indicates the difficulty in engineering for pathogen immunity.

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Author's contributions KK developed the study, wrote the manuscript and did the antifungal challenge experiments with DL and RP. MAG and RJ did the Western blot. PO provided the two chitinase genes. KR and JC provided the CPO gene that JC had subcloned for bombardment.

Compliance with ethical standards

Conflict of interest None of the authors have any conflict of interest with this research.

References

Chakrabarti A, Ganapathi TR, Mukherjee PK, Bapat VA (2003) MSI-99, a magainin analogue, imparts enhanced disease resistance in transgenic tobacco and banana. Planta 216:587–596

Church GM, Gilbert W (1984) Genome sequencing. Proc Natl Acad Sci USA 81:1991–1995

Cletus J, Balasubramanian V, Vashisht D, Sakthivel N (2013) Transgenic expression of plant chitinases to enhance disease resistance. Biotechnol Lett 35:1719–1732

Collinge DB, Jørgensen HJL, Lund OS, Lyngkjær MF (2010) Engineering pathogen resistance in crop plants: current trends and future prospects. Annu Rev Phytopathol 48:269–291

Dana de las Mercedes M, Pintor-Toro JA, Cubero B (2006) Transgenic tobacco plants overexpressing chitinases of fungal origin show enhanced resistance to biotic and abiotic stress agents. Plant Physiol 142:722–730

Dellaporta S, Wood J, Hicks J (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1:19–21

Di R, Blechl A, Dill-Macky R, Tortora A, Tumer NE (2010) Expression of a truncated form of yeast ribosomal protein L3 in transgenic wheat improves resistance to Fusarium head blight. Plant Sci 178:374–380

Distefano G, La Malfa S, Vitale A, Lorito M, Deng Z, Gentile A (2008) Defence-related gene expression in transgenic lemon plants producing an antimicrobial *Trichoderma harzianum* endochitinase during fungal infection. Trans Res 17:873–879

Emani C, Garcia JM, Lopata-Finch E, Pozo MJ, Uribe P, Kim D-J, Sunilkumar G, Cook DR, Kenerley CM, Rathore KS (2003)

Enhanced fungal resistance in transgenic cotton expressing an endochitinase gene from *Trichoderma virens*. Plant Biotechnol J 1:321–336

Gaspar YM, McKenna JA, McGinness BS, Hinch J, Poon S, Connelly AA, Anderson MA, Heath RL (2014) Field resistance to *Fusarium oxysporum* and *Verticillium dahliae* in transgenic cotton expressing the plant defensin *NaD1*. J Exp Bot 65:1541–1550

Gentile A, Deng Z, La Malfa S, Distefano G, Domina F, Vitale A, Polizzi G, Lorito M, Tribulato E (2007) Enhanced resistance to *Phoma tracheiphila* and *Botrytis cinerea* in transgenic lemon plants expressing a *Trichoderma harzianum* chitinase gene. Plant Breed 126:146–151

Ghag SB, Shekhawat UKS, Ganapathi TR (2012) *Petunia* floral defensins with unique prodomains as novel candidates for development of Fusarium wilt resistance in transgenic banana plants. PLoS ONE 7:e39557. doi:10.1371/journal.pone0039557

Ghag SB, Shekhawat UKS, Ganapathi TR (2014) Native cell-death genes as candidates for developing wilt resistance in transgenic banana plants. AoB Plants 6:plu037. doi:10.1093/aobpla/plu037

Girhepuje PV, Shinde GB (2011) Transgenic tomato plants expressing a wheat endochitinase gene demonstrate enhanced resistance to *Fusarium oxysporum* f. sp. l*ycopersici*. Plant Cell, Tissue Organ Cult 105:243–251

Han J, Lakshman DK, Galvez LC, Mitra S, Baenziger PS, Mitra A (2012) Transgenic expression of lactoferrin imparts enhanced resistance to head blight of wheat caused by Fusarium graminearum. BMC Plant Biol 12:33. http://www.biomedicentral.com/1471-2229/12/33

Hartl L, Zach S, Seidl-Seiboth V (2012) Fungal chitinases: diversity, mechanistic properties and biotechnological potential. Appl Microbiol Biotechnol 93:533–543

Jacks TJ, de Lucca AJ, Rajasekaran K, Stromberg K, van Pée KH (2000) Antifungal and peroxidative activities of nonheme chloroperoxidase in relation to transgenic plant protection. J Agric Food Chem 48:4561–4564

Jacks TJ, Rajasekaran K, Stromberg KD, de Lucca AJ, van Pée KH (2002) Evaluation of peracid formation as the basis for resistance to infection in plants transformed with haloperoxidase. J Agric Food Chem 50:706–709

Jongedijk E, Tigelaar H, van Roekel JSC, Bres-Vloemans SA, Dekker I, van den Elzen PJM, Cornelissen BJC, Melchers LS (1995) Synergistic activity of chitinases and β-1,3-glucanases enhances fungal resistance in transgenic tomato plants. Euphytica 85:173–180

Kamo K, Lakshman D, Bauchan G, Rajasekaran K, Cary J, Jaynes J (2015) Expression of a synthetic antimicrobial peptide, D4E1, in Gladiolus plants for resistance to *Fusarium oxysporum* f. sp. *gladioli*. Plant Cell Tissue Organ Cult 121:459–467

Kumar V, Parkhi V, Kenerley CM, Rathore KS (2009) Defense-related gene expression and enzyme activities in transgenic cotton plants expressing an endochitinase gene from *Trichoderma virens* in response to interaction with *Rhizoctonia solani*. Planta 230:277–291

Kumar DP, Singh RK, Anupama PD, Solanki MK, Kumar S, Srivastava AK, Singhal PD, Arora DK (2011) Studies on exochitinase production from *Trichoderma asperellum* UTP-16 and its characterization. Ind J Microbiol 52:388–395

Larone DH (1995) Medically important fungi, a guide to identification, 3rd edn. American Society for Microbiology Press, Washington

Li WL, Faris JD, Muthukrishnan S, Liu DJ, Chen PD, Gill BS (2001) Isolation and characterization of novel cDNA clones of acidic chitinases and β-1,3-glucanases from wheat spikes infected by *Fusarium graminearum*. Theor Appl Genet 102:353–362

Li HP, Zhang JB, Shi RP, Huang T, Fischer R, Liao YC (2008) Engineering *Fusarium* head blight resistance in wheat by expression of a fusion protein containing a *Fusarium*-specific antibody and an antifungal peptide. Mol Plant Microbe Interact 21:1242–1248

Li Z, Zhou M, Zhang Z, Ren L, Du L, Zhang B, Xu H, Xin Z (2011) Expression of a radish defensing in transgenic wheat confers increased resistance to *Fusarium graminearum* and *Rhizoctonia cerealis*. Funct Integr Genomics 11:63–70

Lorito M, Woo SL, Fernandez IG, Colucci G, Harman GE, Pintor-Toro JA, Filippone E, Muccifora S, Lawrence CB, Zoina A, Tuzun S, Scala F (1998) Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. Proc Natl Acad Sci USA 95:7860–7865

Makandar R, Essig JS, Schapaugh MA, Trick HN, Shah J (2006) Genetically engineered resistance to Fusarium head blight in wheat by expression of *Arabidopsis NPR1*. MPMI 19:123–129

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp 368–369

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Niu C, Akasaka-Kennedy Y, Faustinelli P, Joshi M, Rajasekaran K, Yang H, Chu Y, Cary J, Ozias-Akins P (2009) Antifungal activity in transgenic peanut (*Arachis hypogaea* L.) conferred by a nonheme chloroperoxidase gene. Peanut Sci 36:126–132

Ntui VO, Thirukkumaran G, Azadi P, Khan RS, Nakamura I, Mii M (2010) Stable integration and expression of wasabi defensing gene in "Egusi' melon (*Colocynthis citrullus* L.) confers resistance to Fusarium wilt and Alternaria leaf spot. Plant Cell Rep 29:943–954

Okubara PA, Dickman MB, Blechl AE (2014) Molecular and genetic aspects of controlling the soilborne necrotrophic pathogens *Rhizoctonia* and *Pythium*. Plant Sci 228:61–70

Paul J-Y, Becker DK, Dickman MB, Harding RM, Khanna HK, Dale JL (2011) Apoptosis-related genes confer resistance to Fusarium wilt in transgenic 'Lady Finger' bananas. Plant Biotech J 9:1141–1148

Popowich EA, Firsov AP, Mitiouchkina TY, Filipenya VL, Dolgov SV, Reshetnikov VN (2007) *Agrobacterium*-mediated transformation of *Hyacinthus orientalis* with thaumatin II gene to control fungal diseases. Plant Cell, Tissue Organ Cult 90:237–244

Rajasekaran K, Cary JW, Jacks TJ, Stromberg KD, Cleveland TE (2000) Inhibition of fungal growth in planta and in vitro by transgenic tobacco expressing a bacterial nonheme chloroperoxidase gene. Plant Cell Rep 19:333–338

Ruhlman TA, Rajasekaran K, Cary JW (2014) Expression of chloroperoxidase from *Pseudomonas pyrrocinia* in tobacco plastids for fungal resistance. Plant Sci 228:98–106

Sanford J, Smith FD, Russell JA (1993) Optimizing the biolistic process for different biological applications. Methods Enzymol 217:483–510

Shin S, Mackintosh CA, Lewis J, Heinen SJ, Radmer L, Dill-Macky R, Baldridge GD, Zeyen RJ, Muehlbauer GJ (2008) Transgenic wheat expressing a barley class II chitinase gene has enhanced resistance against *Fusarium graminearum*. J Exp Bot 59:2371–2378

Somssich IE, Bollmann J, Hahlbrock K, Kombrink E, Schulz W (1989) Differential early activation of defense-related genes in elicitor-treated parsley cells. Plant Mol Biol 12:227–234

van Pée K-H (1996) Biosynthesis of halogenated metabolites by bacteria. Ann Rev Microbiol 50:375–399

Volpi C, Janni M, Lionetti V, Bellincampi D, Favaron F, D'Ovidio R (2011) The ectopic expression of a pectin methyl esterase

inhibitor increases pectin methyl esterification and limits fungal diseases in wheat. MPMI 24:1012–1019

Wiesner W, van Pee K-H, Lingens F (1986) Detection of a new chloroperoxidase in *Pseudomonas pyrrocinia*. FEBS Lett 209:321–324

Wilfret GJ (1992) Gladiolus. In: Larson RA (ed) Introduction to floriculture. Academic Press, San Diego, pp 143–157

Wolfgang W, van Pee K-H, Lingens F (1986) Detection of a new chloroperoxidase in *Pseudomonas pyrrocinia*. FEBS 209: 321–324

Wolframm C, Lingens F, Mutzel R, van Pée K-H (1993) Chloroperoxidase-encoding gene from *Pseudomonas pyrrocinia*: sequence expression in heterologous hosts, and purification of the enzyme. Gene 130:131–135

Wu L, Zhang H, Zhang J, Wu L, Xi Z, Chen Y (2015) Overexpression of Zm-HINT1 in *Arabidopsis thaliana* enhances resistance to *Fusarium graminearum*. Plant Cell, Tissue Organ Cult 121: 511–518